

PB-0009-1CIP

**CARDIAC MUSCLE-ASSOCIATED GENES**

This application is a continuation-in-part of USSN 09/299,708, filed 26 April 1999.

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**FIELD OF THE INVENTION**

The invention relates to 48 polynucleotides associated with cardiac muscle function that were identified by their coexpression with known cardiac muscle-associated genes. The invention also relates to the use of these polynucleotides, their encoded proteins and antibodies which specifically bind the proteins in diagnosis, prognosis, treatment, and evaluation of therapies for disorders associated with cardiac muscle function.

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**BACKGROUND OF THE INVENTION**

Vertebrates have three classes of muscle: skeletal, smooth, and cardiac. Skeletal and cardiac muscles have a striped appearance in the light microscope and are therefore called striated. Cardiac muscle resembles skeletal muscle in many respects, but it is specialized for the continuous, involuntary, rhythmic contractions needed for pumping blood. Smooth muscles lack striations and surround internal organs such as the intestines, the uterus, and large blood vessels. Skeletal muscle is under the voluntary control of the nervous system. Cardiac muscle and smooth muscle are under the involuntary control of the nervous system. Compared with striated muscles, smooth muscle cells contract and relax slowly and can create and maintain tension for long periods of time.

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Muscle tissue is composed of bundles of multinucleated muscle cells (myofibers). Each muscle cell contains bundles of actin and myosin filaments (myofibrils) which extend the length of the cell. The myofibril is composed of a chain of sarcomeres. The sarcomere is the functional unit of contraction. Myosin filaments are sandwiched between alternating layers of actin filaments. Myosin filaments are composed of heavy and light chain proteins. Actin filaments are capped by two proteins, capZ and tropomodulin. In addition, the myosin-binding sites of actin filaments are protected by the tropomyosin-troponin regulatory complex. Contraction of muscle is initiated by action potential-stimulated release from the sarcoplasmic reticulum of calcium ions into the cell to levels greater than  $10^{-6}$  M. Binding of calcium ions to troponin causes tropomyosin to move towards the center of the actin filament. This movement exposes the myosin-binding sites of actin. Prior to contraction, the N-terminal domain of the myosin heavy chain-light chain complex (myosin head) forms a cross-bridge with actin filaments. Binding of ATP to the myosin head causes dissociation of myosin from actin. This is followed by a conformational change of the myosin head and hydrolysis of ATP. The myosin head then forms a new cross-bridge with actin filaments. Successive cycle of ATP-binding, dissociation from actin, conformational changes, ATP hydrolysis, and crossbridge formation results in muscle contraction. Relaxation is initiated when calcium ion levels in the cell fall below  $10^{-6}$  M. At

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that level, calcium ions dissociate from troponin, which then shields the myosin-binding sites of actin.

Gap junctions, very permeable parts of the cell membrane, connect individual muscle cells with each other. Through these gap junctions, ions diffuse relatively freely and transmit action potentials to all muscle cells.

Differentiation of muscle cells during embryogenesis and ontogeny is regulated by a number of nuclear transcription factors such as myogenin, MyoD, MEF2A, and myf-5, and by cell cycle proteins such as p21, p57, and RB. Expression of the genes which encode some of these myogenic regulatory proteins has been correlated with certain type of tumor and other disorders (Wang *et al.* (1995) *Am J Pathol* 147:1799-1810; Miyagawa *et al.* (1998) *Nat Genet* 18:15-17; and Sedehizade *et al.* (1997) *Muscle Nerve* 20:186-194).

Contemporary techniques for diagnosis of cardiac muscle abnormalities rely mainly on observation of clinical symptoms, electrocardiograms, and serological analyses of metabolites and enzymes. Relatively mild symptoms in the earlier stages of heart disease may even be overlooked. In addition, the serological analyses of the limited number of hormones or peptides do not always differentiate among those diseases or syndromes which have overlapping or near-normal ranges of hormonal or marker protein levels. Thus, development of new techniques, such as microarrays and transcript imaging, will contribute to the early and accurate diagnosis or to a better understanding of molecular pathogenesis of cardiac disorders.

The present invention satisfies a need in the art by providing new compositions that are useful for diagnosis, prognosis, treatment, and evaluation of therapies for disorders associated with cardiac muscle function.

**SUMMARY OF THE INVENTION**

The invention provides a composition comprising a plurality of polynucleotides having the nucleic acid sequences of SEQ ID NOs:1-48 that are highly significantly co-expressed with known the cardiac muscle-associated genes: atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin.

The invention also provides an isolated polynucleotide comprising a nucleic acid sequence selected from SEQ ID NOs:1-48 and the complements thereof. In different aspects, the polynucleotide is used as a surrogate marker, as a probe, in an expression vector, and in the diagnosis, prognosis, evaluation of therapies and treatment of disorders such as atherosclerosis, arteriosclerosis, atrial fibrillation, cancer (myxoma) and complications of cancer, cardiac injury, congestive heart failure, coronary artery disease, hypertension, hypertrophic cardiomyopathy, myocardial hypertrophy, myocardial infarction, and plaque. The invention further provides a composition comprising a polynucleotide and a labeling moiety.

The invention provides a method for using a composition or a polynucleotide to screen a plurality of

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molecules and compounds to identify or to purify ligands which specifically bind to the composition or the polynucleotide. The molecules are selected from DNA molecules, RNA molecules, peptide nucleic acids, peptides, mimetics, ribozymes, transcription factors, enhancers, and repressors.

The invention provides a method for using a composition or a polynucleotide to detect gene expression in a sample by hybridizing the composition or polynucleotide to nucleic acids of the sample under conditions for formation of one or more hybridization complexes and detecting hybridization complex formation, wherein complex formation indicates gene expression in the sample. In one aspect, the composition or polynucleotide is attached to a substrate. In another aspect, the nucleic acids of the sample are amplified prior to hybridization. In yet another aspect, complex formation is compared with at least one standard and indicates the presence of a disorder.

The invention provides a purified protein or a portion thereof selected from SEQ ID NOs:49-62, which is encoded by a polynucleotide that is highly significantly co-expressed with genes known to be involved in disorders associated with cardiac muscle function. The invention also provides a method for using a protein to screen a plurality of molecules to identify or to purify at least one ligand which specifically binds the protein. The molecules are selected from aptamers, DNA molecules, RNA molecules, peptide nucleic acids, peptides, mimetics, ribozymes, proteins, antibodies, agonists, antagonists, immunoglobulins, inhibitors, pharmaceutical agents or drug compounds.

The invention provides a method of using a protein to make an antibody comprising immunizing a animal with the protein under conditions to elicit an antibody response, isolating animal antibodies, attaching the protein to a substrate, contacting the substrate with isolated antibodies under conditions to allow specific binding to the protein, and dissociating the antibodies from the protein, thereby obtaining purified antibodies. The invention also provides a method for using the antibody to detect expression of a protein in a sample, the method comprising combining the antibody with a sample under conditions which allow the formation of antibody:protein complexes, and detecting complex formation, wherein complex formation indicates expression of the protein in the sample. The invention also provides a composition comprising a polynucleotide, a protein, or an antibody that specifically binds a protein and a labeling moiety or a pharmaceutical carrier.

**BRIEF DESCRIPTION OF THE SEQUENCE LISTING AND TABLES**

The Sequence Listing provides exemplary polynucleotide sequences, SEQ ID NOs:1-48, and polypeptide sequences, SEQ ID NOs:49-62. Each sequence is identified by a sequence identification number (SEQ ID NO) and by the Incyte clone number with which the sequence was first identified.

Table 1 presents the results of co-expression analysis. The entries in the table are the p-values which link the novel polynucleotides with known marker genes.

Table 2 shows the characterization of proteins having the amino acid sequences of SEQ ID NO:49-62.

## DESCRIPTION OF THE INVENTION

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality of such host cells, and a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

## DEFINITIONS

"Markers" refer to polynucleotides, proteins, and antibodies which are useful in the diagnosis, prognosis, evaluation of therapies and treatment of disorders associated with cardiac muscle function.

Typically, this means that the marker gene or polynucleotide is differentially expressed in samples from subjects predisposed to, manifesting, or diagnosed with disorders associated with cardiac muscle function.

"Differential expression" refers to an increased or up-regulated or a decreased or down-regulated expression as detected by presence, absence or at least about a two-fold change in the amount of transcribed messenger RNA or protein in a sample.

"Disorders associated with cardiac muscle function" specifically include, but are not limited to, the following conditions, diseases, and disorders: atherosclerosis, arteriosclerosis, atrial fibrillation, cancer (myxoma) and complications of cancer, cardiac injury, congestive heart failure, coronary artery disease, hypertension, hypertrophic cardiomyopathy, myocardial hypertrophy, myocardial infarction, and plaque.

"Isolated or purified" refers to a polynucleotide or protein that is removed from its natural environment and that is separated from other components with which it is naturally present.

"Genes known to be highly, and differentially, expressed in cardiac muscle function" which were used in the co-expression analysis included atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin.

"Polynucleotide" refers to an isolated cDNA. It can be of genomic or synthetic origin, double-stranded or single-stranded, and combined with vitamins, minerals, carbohydrates, lipids, proteins, or other nucleic acids to perform a particular activity or form a useful composition.

"Protein" refers to a purified polypeptide whether naturally occurring or synthetic.

"Sample" is used in its broadest sense. A sample containing nucleic acids can comprise a bodily fluid; an extract from a cell; a chromosome, organelle, or membrane isolated from a cell; genomic DNA, RNA, or cDNA in solution or bound to a substrate; a cell; a tissue; a tissue print; and the like.

"Substrate" refers to any rigid or semi-rigid support to which polynucleotides or proteins are bound and includes membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, capillaries or

other tubing, plates, polymers, and microparticles with a variety of surface forms including wells, trenches, pins, channels and pores.

A "transcript image" is a profile of gene transcription activity in a particular tissue at a particular time.

A "variant" refers to a polynucleotide or protein whose sequence diverges from about 5% to about 30% from the nucleic acid or amino acid sequences of the Sequence Listing.

## THE INVENTION

The present invention employed "guilt by association (GBA)", a method for using marker genes known to be associated with cardiac muscle function to identify surrogate markers, polynucleotides that are similarly associated or co-expressed in the same tissues, pathways or disorders (Walker and Volkmuth (1999) Prediction of gene function by genome-scale expression analysis: prostate-associated genes. *Genome Res* 9:1198-1203, incorporated herein by reference). The genes known to be associated with cardiac muscle function are atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin. In particular, the method identifies cDNAs cloned from mRNA transcripts which were active in tissues removed from subjects with cardiac disorders including, but not limited to, atherosclerosis, arteriosclerosis, atrial fibrillation, cancer (myxoma) and complications of cancer, cardiac injury, congestive heart failure, coronary artery disease, hypertension, hypertrophic cardiomyopathy, myocardial hypertrophy, myocardial infarction, and plaque. The polynucleotides, their encoded proteins and antibodies which specifically bind to the encoded proteins are useful in the diagnosis, prognosis, evaluation of therapies, and treatment of disorders associated with cardiac muscle function. USSN 09/299,708 is incorporated in its entirety by reference herein.

Guilt by association provides for the identification of polynucleotides that are expressed in a plurality of libraries. The polynucleotides represent genes of unknown function which are co-expressed in a specific pathway, disease process, subcellular compartment, cell type, tissue, or species. The expression patterns of the genes known to be highly and differentially expressed during cardiac muscle function; atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin; are compared with those of polynucleotides with unknown function to determine whether a specified co-expression probability threshold is met. Through this comparison, a subset of the polynucleotides having a high co-expression probability with the known marker genes can be identified.

The polynucleotides originate from human cDNA libraries. These polynucleotides can also be selected from a variety of sequence types including, but not limited to, expressed sequence tags (ESTs), assembled polynucleotides, full length coding regions, and 3' untranslated regions. To be considered in GBA or co-

expression analysis, the polynucleotides had to have been expressed in at least five cDNA libraries. In this application, GBA was applied to a total of 45,233 assembled polynucleotide bins that met the criteria of having been expressed in at least five libraries.

The cDNA libraries used in the co-expression analysis were obtained from adrenal gland, biliary tract, bladder, blood cells, blood vessels, bone marrow, brain, bronchus, cartilage, chromaffin system, colon, connective tissue, cultured cells, embryonic stem cells, endocrine glands, epithelium, esophagus, fetus, ganglia, heart, hypothalamus, hemic/immune system, intestine, islets of Langerhans, kidney, larynx, liver, lung, lymph, muscles, neurons, ovary, pancreas, penis, phagocytes, pituitary, placenta, pleura, prostate, salivary glands, seminal vesicles, skeleton, spleen, stomach, testis, thymus, tongue, ureter, uterus, and the like. The number of cDNA libraries analyzed can range from as few as three to greater than 10,000 and preferably, the number of the cDNA libraries is greater than 500.

In a preferred embodiment, the polynucleotides are assembled from related sequences, such as sequence fragments derived from a single transcript. Assembly of the polynucleotide can be performed using sequences of various types including, but not limited to, ESTs, extension of the EST, shotgun sequences from a cloned insert, or full length cDNAs. In a most preferred embodiment, the polynucleotides are derived from human sequences that have been assembled using the algorithm disclosed in USSN 9,276,534, filed March 25, 1999, and used in USSN 09/226,994, filed 7 January 1999, both incorporated herein by reference.

Experimentally, differential expression of the polynucleotides can be evaluated by methods including, but not limited to, differential display by spatial immobilization or by gel electrophoresis, genome mismatch scanning, representational difference analysis, and transcript imaging. For example, the results of transcript imaging for SEQ ID NOs:29 and 44 are shown in Example IX. Differential expression of SEQ ID NO:29 is highly specifically correlated with hypertension, and SEQ ID NO:44, with myocardial infarction. The transcript image provided direct confirmation of the strength of co-expression analysis--the use of known genes to identify unknown polynucleotides and their encoded proteins which are highly significantly associated with disorders associated with cardiac muscle function. Additionally, differential expression can be assessed by microarray technology. These methods can be used alone or in combination.

Genes known to be highly expressed in disorders associated with cardiac muscle function can be selected based on research in which the genes are found to be key elements of biochemical or signaling pathways or on the known use of the genes as diagnostic or prognostic markers or therapeutic targets for such disorders. Preferably, the known genes are atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin.

The procedure for identifying novel polynucleotides that exhibit a statistically significant co-

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expression pattern with known genes is as follows. First, the presence or absence of a polynucleotide in a cDNA library is defined: a polynucleotide is present in a cDNA library when at least one cDNA fragment corresponding to the polynucleotide is detected in a cDNA from that library, and a polynucleotide is absent from a library when no corresponding cDNA fragment is detected.

5           Second, the significance of co-expression is evaluated using a probability method to measure a due-to-chance probability of the co-expression. The probability method can be the Fisher exact test, the chi-squared test, or the kappa test. These tests and examples of their applications are well known in the art and can be found in standard statistics texts (Agresti (1990) Categorical Data Analysis, John Wiley & Sons, New York NY; Rice (1988) Mathematical Statistics and Data Analysis, Duxbury Press, Pacific Grove CA). A Bonferroni  
10 correction (Rice, supra, p. 384) can also be applied in combination with one of the probability methods for correcting statistical results of one polynucleotide versus multiple other polynucleotides. In a preferred embodiment, the due-to-chance probability is measured by a Fisher exact test, and the threshold of the due-to-chance probability is set preferably to less than 0.001, more preferably to less than 0.00001.

For example, to determine whether two genes, A and B, have similar co-expression patterns, occurrence data vectors can be generated as illustrated in the table below. The presence of a gene occurring at least once in a library is indicated by a one, and its absence from the library, by a zero.

	Library 1	Library 2	Library 3	...	Library N
Gene A	1	1	0	...	0
Gene B	1	0	1	...	0

For a given pair of genes, the occurrence data in the table above can be summarized in a 2 x 2 contingency table. The second table (below) presents co-occurrence data for gene A and gene B in a total of 30 libraries. Both gene A and gene B occur 10 times in the libraries.

	Gene A Present	Gene A Absent	Total
Gene B Present	8	2	10
Gene B Absent	2	18	20
Total	10	20	30

The second table summarizes and presents: 1) the number of times gene A and B are both present in a library; 2) the number of times gene A and B are both absent in a library; 3) the number of times gene A is present, and gene B is absent; and 4) the number of times gene B is present, and gene A is absent. The upper

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left entry is the number of times the two genes co-occur in a library, and the middle right entry is the number of times neither gene occurs in a library. The off diagonal entries are the number of times one gene occurs, and the other does not. Both A and B are present eight times and absent 18 times. Gene A is present, and gene B is absent, two times; and gene B is present, and gene A is absent, two times. The probability ("p-value") that the above association occurs due to chance as calculated using a Fisher exact test is 0.0003.

This method of estimating the probability for co-expression makes several assumptions. The method assumes that the libraries are independent and are identically sampled. However, in practical situations, the selected cDNA libraries are not entirely independent, because more than one library can be obtained from a single subject or tissue. Nor are they entirely identically sampled, because different numbers of cDNAs can have been sequenced from each library. The number of cDNAs sequenced typically ranges from 5,000 to 10,000 cDNAs per library. After the Fisher exact co-expression probability is calculated for each polynucleotide versus all other assembled polynucleotides that occur, a Bonferroni correction for multiple statistical tests is applied.

Using the method of the present invention, we have identified polynucleotides, SEQ ID NOs:1-48 and their encoded proteins, SEQ ID NOs:49-62, that exhibit highly significant co-expression probability with known marker genes for disorders associated with cardiac muscle function. The results presented in Example VI show the direct associations among the novel polynucleotides and the known marker genes for disorders associated with cardiac muscle function. Therefore, by these associations, the novel polynucleotides are useful as surrogate markers for the co-expressed known markers in diagnosis, prognosis, evaluation of therapies and treatment of disorders associated with cardiac muscle function. Further, the proteins or peptides expressed from the novel polynucleotides are either potential therapeutics or targets for the identification and/or development of therapeutics.

In one embodiment, the present invention encompasses a composition comprising a plurality of polynucleotides having the nucleic acid sequences of SEQ ID NOs:1-48 or the complements thereof. These 48 polynucleotides are shown by the method to have significant co-expression with known markers for disorders associated with cardiac muscle function. The invention also provides a polynucleotide, its complement, a probe comprising the polynucleotide or the complement thereof selected from SEQ ID NOs:1-48.

The polynucleotide can be used to search against the GenBank primate (pri), rodent (rod), mammalian (mam), vertebrate (vrtp), and eukaryote (eukp) databases; the encoded protein, against GenPept, SwissProt, BLOCKS (Bairoch *et al.* (1997) *Nucleic Acids Res* 25:217-221), PFAM, and other databases that contain previously identified and annotated protein sequences, motifs, and gene functions. Methods that search for primary sequence patterns with secondary structure gap penalties (Smith *et al.* (1992) *Protein Engineering* 5:35-51) as well as algorithms such as Basic Local Alignment Search Tool (BLAST; Altschul (1993) *J Mol*



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Evol 36:290-300; Altschul et al. (1990) J Mol Biol 215:403-410), BLOCKS (Henikoff and Henikoff (1991) Nucleic Acids Res 19:6565-6572), Hidden Markov Models (HMM; Eddy (1996) Cur Opin Str Biol 6:361-365; Sonnhammer et al. (1997) Proteins 28:405-420), and the like, can be used to manipulate and analyze nucleotide and amino acid sequences. These databases, algorithms and other methods are well known in the art and are described in Ausubel et al. (1997; Short Protocols in Molecular Biology, John Wiley & Sons, New York NY, unit 7.7) and in Meyers (1995; Molecular Biology and Biotechnology, Wiley VCH, New York NY, p 856-853).

Also encompassed by the invention are polynucleotides that are capable of hybridizing to SEQ ID NOs:1-48 and the complements thereof under highly stringent conditions. Stringency can be defined by salt concentration, temperature, and other chemicals and conditions well known in the art. Conditions can be selected, for example, by varying the concentrations of salt in the prehybridization, hybridization, and wash solutions or by varying the hybridization and wash temperatures. With some substrates, the temperature can be decreased by adding a solvent such as formamide to the prehybridization and hybridization solutions.

Hybridization can be performed at low stringency, with buffers such as 5xSSC (saline sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 60C, which permits complex formation between two nucleic acid sequences that contain some mismatches. Subsequent washes are performed at higher stringency with buffers such as 0.2xSSC with 0.1% SDS at either 45C (medium stringency) or 68C (high stringency), to maintain hybridization of only those complexes that contain completely complementary sequences. Background signals can be reduced by the use of detergents such as SDS, sarcosyl, or TRITON X-100 (Sigma-Aldrich, St. Louis MO), and/or a blocking agent, such as salmon sperm DNA. Hybridization methods are described in detail in Ausubel (supra, units 2.8-2.11, 3.18-3.19 and 4-6-4.9) and Sambrook et al. (1989; Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

A polynucleotide can be extended utilizing primers and employing various PCR-based methods known in the art to detect upstream sequences such as promoters and other regulatory elements. (See, e.g., Dieffenbach and Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY.) Commercially available kits such as XL-PCR (Applied Biosystems (ABI), Foster City CA), cDNA libraries (Life Technologies, Rockville MD) or genomic libraries (Clontech, Palo Alto CA) and nested primers can be used to extend the sequence. For all PCR-based methods, primers can be designed using commercially available software (e.g., LASERGENE software, DNASTAR, Madison WI or another program), to be about 15 to 30 nucleotides in length, to have a GC content of about 50%, and to form a hybridization complex at temperatures of about 68C to 72C.

In another aspect of the invention, the polynucleotide can be cloned into a recombinant vector that directs the expression of the protein, or structural or functional portions thereof, in host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode functionally equivalent amino

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acid sequence can be produced and used to express the protein encoded by the polynucleotide. The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter the nucleotide sequences for a variety of purposes including, but not limited to, modification of the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation, as described in USPN 5,830,721, and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, oligonucleotide-mediated site-directed mutagenesis can be used to introduce mutations that create new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, and so forth.

In order to express a biologically active protein, the polynucleotide or derivatives thereof, can be inserted into an expression vector with elements for transcriptional and translational control of the inserted coding sequence in a particular host. These elements include regulatory sequences, such as enhancers, constitutive and inducible promoters, and 5' and 3' untranslated regions. Methods which are well known to those skilled in the art can be used to construct such expression vectors. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination (Ausubel, supra, unit 16).

A variety of expression vector/host cell systems can be utilized to express the polynucleotide. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with baculovirus vectors; plant cell systems transformed with viral or bacterial expression vectors; or animal cell systems. For long term production of recombinant proteins in mammalian systems, stable expression in cell lines is preferred. For example, the polynucleotide can be transformed into cell lines using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable or visible marker gene on the same or on a separate vector. The invention is not to be limited by the vector or host cell employed.

In general, host cells that contain the polynucleotide and that express the protein can be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations, PCR amplification, and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or amino acid sequences. Immunological methods for detecting and measuring the expression of the protein using either specific polyclonal or monoclonal antibodies are known in the art. Examples of such techniques include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and fluorescence activated cell sorting (FACS).

Host cells transformed with the polynucleotide can be cultured under conditions for the expression and

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recovery of the protein from cell culture. The protein produced by a transgenic cell can be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing the polynucleotide can be designed to contain signal sequences which direct secretion of the protein through a prokaryotic cell wall or eukaryotic cell membrane.

5 In addition, a host cell strain can be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the protein include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein can also be used to specify protein targeting, folding, and/or activity. Different host cells which have specific cellular machinery and  
10 characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38) are available from the ATCC (Manassas VA) and can be chosen to ensure the correct modification and processing of the expressed protein.

In another embodiment of the invention, natural, modified, or recombinant polynucleotides are ligated to a heterologous sequence resulting in translation of a fusion protein containing heterologous protein moieties in any of the aforementioned host systems. Such heterologous protein moieties facilitate purification of fusion proteins using commercially available affinity matrices. Such moieties include, but are not limited to, glutathione S-transferase, maltose binding protein, thioredoxin, calmodulin binding peptide, 6-His, FLAG, c-myc, hemagglutinin, and monoclonal antibody epitopes.

In another embodiment, the polynucleotides, wholly or in part, are synthesized using chemical or enzymatic methods well known in the art (Caruthers et al. (1980) Nucl Acids Symp Ser (7) 215-233; Ausubel, supra, units 10.4 and 10.16). Peptide synthesis can be performed using various solid-phase techniques (Roberge et al. (1995) Science 269:202-204), and machines such as the ABI 431A peptide synthesizer (ABI) can be used to automate synthesis. If desired, the amino acid sequence can be altered during synthesis to produce a more stable variant for therapeutic use.

#### **25 SCREENING, DIAGNOSTICS AND THERAPEUTICS**

The polynucleotides can be used as surrogate markers in diagnosis, prognosis, evaluation of therapies and treatment of disorders associated with cardiac muscle function including, but not limited to, atherosclerosis, arteriosclerosis, atrial fibrillation, cancer (myxoma) and complications of cancer, cardiac injury, congestive heart failure, coronary artery disease, hypertension, hypertrophic cardiomyopathy,  
30 myocardial hypertrophy, myocardial infarction, and plaque.

The polynucleotide can be used to screen a plurality or library of molecules and compounds for specific binding affinity. The assay can be used to screen DNA molecules, RNA molecules, peptide nucleic acids, peptides, mimetics, ribozymes, or proteins including transcription factors, enhancers, repressors, and the

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like which regulate the activity of the polynucleotide in the biological system. The assay involves providing a plurality of molecules and compounds, combining a polynucleotide or a composition of the invention with the plurality of molecules and compounds under conditions to allow specific binding, and detecting specific binding to identify at least one molecule or compound which specifically binds at least one polynucleotides of the invention.

Similarly the proteins, or portions thereof, can be used to screen a plurality or library of molecules or compounds in any of a variety of screening assays to identify a ligand. The protein employed in such screening can be free in solution, affixed to an abiotic substrate or expressed on the external, or a particular internal surface, of a bacterial, or other, cell. Specific binding between the protein and the ligand can be measured.

The assay can be used to screen aptamers, DNA molecules, RNA molecules, peptide nucleic acids, peptides, mimetics, ribozymes, proteins, antibodies, agonists, antagonists, immunoglobulins, inhibitors, pharmaceutical agents or drug compounds and the like, which specifically bind the protein. One method for high throughput screening using very small assay volumes and very small amounts of test compound is described in Burbaum *et al.* USPN 5,876,946, incorporated herein by reference, which screens large numbers of molecules for enzyme inhibition or receptor binding.

In one preferred embodiment, the polynucleotides are used for diagnostic purposes to determine the differential expression of a gene in a sample. The polynucleotide consists of complementary RNA and DNA molecules, branched nucleic acids, and/or PNAs. In one alternative, the polynucleotides are used to detect and quantify gene expression in biopsied samples in which differential expression of the polynucleotide indicates the presence of a disorder. In another alternative, the polynucleotide can be used to detect genetic polymorphisms associated with a disease or disorder. In a preferred embodiment, these polymorphisms are detected in an mRNA transcribed from an endogenous gene.

In another preferred embodiment, the polynucleotide is used as a probe. Specificity of the probe is determined by whether it is made from a unique region, a regulatory region, or from a region encoding a conserved motif. Both probe specificity and the stringency of the diagnostic hybridization or amplification will determine whether the probe identifies only naturally occurring, exactly complementary sequences, allelic variants, or related sequences. Probes designed to detect related sequences should preferably have at least 50% sequence identity to at least a fragment of a polynucleotide of the invention.

Methods for producing hybridization probes include the cloning of nucleic acid sequences into vectors for the production of RNA probes. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by adding RNA polymerases and labeled nucleotides. Probes can incorporate nucleotides labeled by a variety of reporter groups including, but not limited to, radionuclides such as <sup>32</sup>P or <sup>35</sup>S, enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling

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systems, fluorescent labels such as Cy3 and Cy5, and the like. The labeled polynucleotides can be used in Southern or northern analysis, dot blot, or other membrane-based technologies, on chips or other substrates, and in PCR technologies. Hybridization probes are also useful in mapping the naturally occurring genomic sequence. Fluorescent in situ hybridization (FISH) can be correlated with other physical chromosome mapping techniques and genetic map data as described in Heinz-Ulrich et al. (In: Meyers, supra, pp. 965-968). In many cases, genomic context helps identify genes that encode a particular protein family. (See, e.g., Kirschning et al. (1997) Genomics 46:416-25.)

The polynucleotide can be labeled using standard methods and added to a sample from a subject under conditions for the formation and detection of hybridization complexes. After incubation the sample is washed, and the signal associated with complex formation is quantitated and compared with at least one standard value. Standard values are derived from any control sample, typically one that is free of the suspect disorder and from one that represents a single, specific and preferably, staged disorder. If the amount of signal in the subject sample is distinguishable from the standards, then differential expression in the subject sample indicates the presence of the disorder. Qualitative and quantitative methods for comparing complex formation in subject samples with previously established standards are well known in the art.

Such assays can also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual subject. Once the presence of the disorder has been established and a treatment protocol is initiated, hybridization, amplification, or antibody assays can be repeated on a regular basis to determine when gene or protein expression in the patient begins to approximate that which is observed in a healthy subject. The results obtained from successive assays can be used to show the efficacy of treatment over a period ranging from several hours, e.g. in the case of toxic shock, to many years, e.g. in the case of osteoarthritis.

The polynucleotides can be used on a substrate such as a microarray to monitor gene expression, to identify splice variants, mutations, and polymorphisms. Information derived from analyses of expression patterns can be used to determine gene function, to understand the genetic basis of a disease, to diagnose a disorder, and to develop and monitor the activities of therapeutic agents used to treat a disorder. Microarrays can also be used to detect genetic diversity, single nucleotide polymorphisms, which may characterize a particular population, at the genomic level.

In another embodiment, antibodies or Fabs comprising an antigen binding site that specifically binds the protein can be used for the diagnosis of diseases characterized by the differential expression of the protein. A variety of protocols for measuring protein expression, including ELISAs, RIAs, FACS and antibody arrays, are well known in the art and provide a basis for diagnosing differential or abnormal levels of expression. Standard values for protein expression parallel those reviewed above for nucleotide expression. The amount of

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complex formation can be quantitated by various methods, preferably by photometric means. Quantities of the protein expressed in subject samples are compared with standard values. Deviation between standard and subject values establishes the parameters for diagnosing or monitoring a particular disorder. Alternatively, one can use competitive drug screening assays in which neutralizing antibodies capable of binding specifically with the protein compete with a test compound. Antibodies can be used to detect the presence of any peptide which shares one or more epitopes or antigenic determinants with the protein. In one aspect, the antibodies of the present invention can be used for treatment of a disorder, delivery of therapeutics, or monitoring therapy during treatment.

In another aspect, the polynucleotide, or its complement, can be used therapeutically for the purpose of expressing mRNA and protein, or conversely to block transcription or translation of the mRNA. Expression vectors can be constructed using elements from retroviruses, adenoviruses, herpes or vaccinia viruses, or bacterial plasmids, and the like. These vectors can be used for delivery of nucleotide sequences to a particular target cell population, tissue, or organ. Methods well known to those skilled in the art can be used to construct vectors to express the polynucleotides or their complements. (See, e.g., Maulik *et al.* (1997) Molecular Biotechnology, Therapeutic Applications and Strategies, Wiley-Liss, New York NY.)

Alternatively, the polynucleotide or its complement, can be used for somatic cell or stem cell gene therapy. Vectors can be introduced *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors are introduced into stem cells taken from the subject, and the resulting transgenic cells are clonally propagated for autologous transplant back into that same subject. Delivery of the polynucleotide by transfection, liposome injections, or polycationic amino polymers can be achieved using methods which are well known in the art. (See, e.g., Goldman *et al.* (1997) *Nature Biotechnology* 15:462-466.) Additionally, endogenous gene expression can be inactivated using homologous recombination methods which insert an inactive gene sequence into the coding region or other targeted region of the genome. (See, e.g. Thomas *et al.* (1987) *Cell* 51: 503-512.)

Vectors containing the polynucleotide can be transformed into a cell or tissue to express a missing protein or to replace a nonfunctional protein. Similarly a vector constructed to express the complement of the polynucleotide can be transformed into a cell to down-regulate protein expression. Complementary or antisense sequences can consist of an oligonucleotide derived from the transcription initiation site; nucleotides between about positions -10 and +10 from the ATG are preferred. Similarly, inhibition can be achieved using triple helix base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature. (See, e.g., Gee *et al.* In: Huber and Carr (1994) Molecular and Immunologic Approaches, Futura Publishing, Mt. Kisco NY, pp. 163-177.)

Ribozymes, enzymatic RNA molecules, can also be used to catalyze the cleavage of mRNA and decrease the levels of particular mRNAs, such as those comprising the polynucleotides of the invention. (See, e.g., Rossi (1994) *Current Biology* 4: 469-471.) Ribozymes can cleave mRNA at specific cleavage sites. Alternatively, ribozymes can cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The construction and production of ribozymes is well known in the art and is described in Meyers (*supra*).

RNA molecules can be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2' O-methyl rather than phosphodiester linkages within the backbone of the molecule. Alternatively, nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases, can be included.

Further, an antagonist, or an antibody that binds specifically to the protein can be administered to a subject to treat a disorders associated with cardiac muscle function. The antagonist, antibody, or fragment can be used directly to inhibit the activity of the protein or indirectly to deliver a therapeutic agent to cells or tissues which express the protein. The therapeutic agent can be a cytotoxic agent selected from a group including, but not limited to, abrin, ricin, doxorubicin, daunorubicin, taxol, ethidium bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphtheria toxin, *Pseudomonas* exotoxin A and 40, radioisotopes, and glucocorticoid.

Antibodies to the protein can be generated using methods that are well known in the art. One method involves immunizing a animal with the protein selected from SEQ ID NOs:49-62 under conditions to elicit an antibody response; isolating animal antibodies; attaching the protein to a substrate; contacting the substrate with isolated antibodies under conditions to allow specific binding to the protein; and dissociating the antibodies from the protein, thereby obtaining purified antibodies. Such antibodies can include, but are not limited to, polyclonal, monoclonal, chimeric, and single chain antibodies, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, such as those which inhibit dimer formation, are especially preferred for therapeutic use. Monoclonal antibodies to the protein can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma, the human B-cell hybridoma, and the EBV-hybridoma techniques. In addition, techniques developed for the production of chimeric antibodies can be used. (See, e.g., Pound (1998) *Immunochemical Protocols*, Methods Mol Biol Vol. 80.) Alternatively, techniques described for the production of single chain antibodies can be employed. Fabs which contain specific binding sites for the protein can also be generated. Various immunoassays can be used to identify antibodies having the

desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art.

Yet further, an agonist of the protein can be administered to a subject to treat a disorder associated with decreased expression, longevity or activity of the protein.

5           An additional aspect of the invention relates to the administration of a pharmaceutical or sterile composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic applications discussed above. Such pharmaceutical compositions can consist of the protein or antibodies, mimetics, agonists, antagonists, or inhibitors of the protein. The compositions can be administered alone or in combination with at least one other agent, such as a stabilizing compound, which can be administered in any  
10       sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a subject alone or in combination with other agents, drugs, or hormones.

          The pharmaceutical compositions utilized in this invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal,  
15       intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

          In addition to the active ingredients, these pharmaceutical compositions can contain pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and  
20       administration can be found in the latest edition of Remington's Pharmaceutical Sciences (Mack Publishing, Easton PA).

          For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models such as mice, rats, rabbits, dogs, or pigs. An animal model can also be used to determine the concentration range and route of administration. Such information can then be used to determine  
25       useful doses and routes for administration in humans.

          A therapeutically effective dose refers to that amount of active ingredient which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity can be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating and contrasting the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population)  
30       statistics. Any of the therapeutic compositions described above can be applied to any subject in need of such therapy, including, but not limited to, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

#### Stem Cells and Their Use



SEQ ID NOs: 1-48 can be useful in the differentiation of stem cells. Eukaryotic stem cells are able to differentiate into the multiple cell types of various tissues and organs and to play roles in embryogenesis and adult tissue regeneration (Gearhart (1998) *Science* 282:1061-1062; Watt and Hogan (2000) *Science* 287:1427-1430). Depending on their source and developmental stage, stem cells can be totipotent with the potential to create every cell type in an organism and to generate a new organism, pluripotent with the potential to give rise to most cell types and tissues, but not a whole organism; or multipotent cells with the potential to differentiate into a limited number of cell types. Stem cells can be transfected with polynucleotides which can be transiently expressed or can be integrated within the cell as transgenes.

Embryonic stem (ES) cell lines are derived from the inner cell masses of human blastocysts and are pluripotent (Thomson *et al.* (1998) *Science* 282:1145-1147). They have normal karyotypes and express high levels of telomerase which prevent senescence and allow the cells to replicate indefinitely. ES cells produce derivatives that give rise to embryonic epidermal, mesodermal and endodermal cells. Embryonic germ (EG) cell lines, which are produced from primordial germ cells isolated from gonadal ridges and mesenteries, also show stem cell behavior (Shamblott *et al.* (1998) *Proc Natl Acad Sci* 95:13726-13731). EG cells have normal karyotypes and appear to be pluripotent.

Organ-specific adult stem cells differentiate into the cell types of the tissues from which they were isolated. They maintain their original tissues by replacing cells destroyed from disease or injury. Adult stem cells are multipotent and under proper stimulation can be used to generate cell types of various other tissues (Vogel (2000) *Science* 287:1418-1419). Hematopoietic stem cells from bone marrow provide not only blood and immune cells, but can also be induced to transdifferentiate to form brain, liver, heart, skeletal muscle and smooth muscle cells. Similarly mesenchymal stem cells can be used to produce bone marrow, cartilage, muscle cells, and some neuron-like cells, and stem cells from muscle have the ability to differentiate into muscle and blood cells (Jackson *et al.* (1999) *Proc Natl Acad Sci* 96:14482-14486). Neural stem cells, which produce neurons and glia, can also be induced to differentiate into heart, muscle, liver, intestine, and blood cells (Kuhn and Svendsen (1999) *BioEssays* 21:625-630; Clarke *et al.* (2000) *Science* 288:1660-1663; Gage (2000) *Science* 287:1433-1438; and Galli *et al.* (2000) *Nature Neurosci* 3:986-991).

Neural stem cells can be used to treat neurological disorders such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis and to repair tissue damaged by strokes and spinal cord injuries. Hematopoietic stem cells can be used to restore immune function in immunodeficient patients or to treat autoimmune disorders by replacing autoreactive immune cells with normal cells to treat diseases such as multiple sclerosis, scleroderma, rheumatoid arthritis, and systemic lupus erythematosus. Mesenchymal stem cells can be used to repair tendons or to regenerate cartilage to treat arthritis. Liver stem cells can be used to repair liver damage. Pancreatic stem cells can be used to replace islet cells to treat diabetes. Muscle stem cells can be used to

regenerate muscle to treat muscular dystrophies (Fontes and Thomson (1999) BMJ 319:1-3; Weissman (2000) Science 287:1442-1446 Marshall (2000) Science 287:1419-1421; and Marmont (2000) Ann Rev Med 51:115-134).

## EXAMPLES

It is to be understood that this invention is not limited to the particular devices, machines, materials and methods described. Although particular embodiments are described, equivalent embodiments can be used to practice the invention. The described embodiments are provided to illustrate the invention and are not intended to limit the scope of the invention which is limited only by the appended claims.

### I cDNA LIBRARY CONSTRUCTION

The cDNA library, LATRNOT01, was selected as an example to demonstrate library construction. The LATRNOT01 cDNA library was constructed from left atrial tissue obtained from a 51-year-old Caucasian female who died of cerebral hemorrhage.

The frozen tissue was homogenized using a pestle and mortar and lysed using a POLYTRON homogenizer (Brinkmann Instruments, Westbury NY) in guanidinium isothiocyanate solution. The lysate was centrifuged over a 5.7 M CsCl cushion using an SW28 swinging bucket rotor in an L8-70M ultracentrifuge (Beckman Coulter, Fullerton CA) for 18 hours at 25,000 rpm and ambient temperature. The RNA was extracted twice with phenol, pH 8.0, precipitated using 0.3 M sodium acetate and 2.5 volumes of ethanol, resuspended in RNase-free water, and treated with DNase at 37C. The mRNA was isolated using the OLIGOTEX kit (Qiagen, Chatsworth CA) and used to construct the cDNA library.

The mRNA was handled according to the recommended protocols in the SUPERScript plasmid system (Life Technologies, Gaithersburg MD). cDNAs were fractionated on a SEPHAROSE CL4B column (Amersham Pharmacia Biotech (APB), Piscataway NJ), and those cDNAs exceeding 400 bp were ligated into the XhoI and EcoRI sites of the  $\lambda$  UNIZAP vector (Stratagene, La Jolla CA). The vector which contained the PBLUESCRIPT phagemid was subsequently transformed into XL1-BLUEMRF host cells (Stratagene). The phagemid forms of individual cDNA clones were obtained by the in vivo excision process, in which the host bacterial strain was co-infected with both the  $\lambda$  library phage and an f1 helper phage. Enzymes derived from both the library-containing and helper phage nicked the  $\lambda$  DNA, initiated new DNA synthesis from defined sequences on the  $\lambda$  target DNA, and created a smaller, single stranded circular phagemid DNA molecule that included all DNA sequences of the PBLUESCRIPT phagemid and the cDNA insert. The phagemid DNA was secreted from the cells, purified, and used to re-infect fresh host cells, where the double stranded phagemid DNA was produced.

### II Isolation and Sequencing of cDNA Clones

Plasmid DNA was released from the bacterial cells and purified using the REAL PREP 96 plasmid kit

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(Qiagen). This kit enabled the simultaneous purification of 96 samples in a 96-well block using multi-channel reagent dispensers. The recommended protocol was employed except for the following changes: 1) the bacteria were cultured in 1 ml of sterile TERRIFIC BROTH (BD Biosciences, San Jose CA) with carbenicillin at 25 mg/L and glycerol at 0.4%; 2) after inoculation, the cells were culture for 19 hours and then lysed in 0.3 ml of lysis buffer; and 3) the plasmid DNA pellet was precipitated in isopropanol and then resuspended in 0.1 ml of distilled water. After the last step in the protocol, samples were transferred to a 96-well block for storage at 4C.

The cDNAs were prepared using a MICROLAB 2200 system (Hamilton, Reno NV) in combination with DNA ENGINE thermal cyclers (MJ Research, Watertown MA). The cDNAs were sequenced by the method of Sanger and Coulson (1975; J Mol Biol 94:441-448) using ABI PRISM 373, 377 or 3700 DNA sequencing systems (ABI). Most of the cDNAs were sequenced using standard ABI protocols and kits at solution volumes of 0.25x - 1.0x. In the alternative, some of the cDNAs were sequenced using solutions and dyes from APB.

### III SELECTION, ASSEMBLY, AND CHARACTERIZATION OF SEQUENCES

The polynucleotides used for co-expression analysis were assembled from EST sequences, 5' and 3' long read sequences, and full length coding sequences. The assembly process is described as follows. EST sequence chromatograms were processed and verified. Quality scores were obtained using PHRED (Ewing *et al.* (1998) Genome Res 8:175-185; Ewing and Green (1998) Genome Res 8:186-194), and edited sequences were loaded into a relational database management system (RDBMS). The sequences were clustered using BLAST with a product score of 50. All clusters of two or more sequences created a bin which represents one transcribed gene.

Assembly of the component sequences within each bin was performed using a modification of Phrap, a publicly available program for assembling DNA fragments (Green, P. University of Washington, Seattle WA). Bins that showed 82% identity from a local pair-wise alignment between any of the consensus sequences were merged.

Bins were annotated by screening the consensus sequence in each bin against public databases, such as GBpri and GenPept from NCBI. The annotation process involved a FASTn screen against the GBpri database in GenBank. Those hits with a percent identity of greater than or equal to 75% and an alignment length of greater than or equal to 100 base pairs were recorded as homolog hits. The residual unannotated sequences were screened by FASTx against GenPept. Those hits with an E value of less than or equal to  $10^{-8}$  were recorded as homolog hits.

Sequences were then reclustered using BLASTn and Cross-Match, a program for rapid amino acid and nucleic acid sequence comparison and database search (Green, *supra*), sequentially. Any BLAST alignment

between a sequence and a consensus sequence with a score greater than 150 was realigned using cross-match. The sequence was added to the bin whose consensus sequence gave the highest Smith-Waterman score (Smith *et al.* (1992) Protein Engineering 5:35-51) amongst local alignments with at least 82% identity. Non-matching sequences were moved into new bins, and assembly processes were repeated.

#### 5 IV HOMOLOGY SEARCHING OF POLYNUCLEOTIDES AND THEIR ENCODED PROTEINS

The polynucleotides of the Sequence Listing or their encoded proteins were used to query databases such as GenBank, SwissProt, BLOCKS, and the like. These databases that contain previously identified and annotated sequences or domains were searched using BLAST or BLAST 2 (Altschul *et al. supra*; Altschul, *supra*) to produce alignments and to determine which sequences were exact matches or homologs. The  
10 alignments were to sequences of prokaryotic (bacterial) or eukaryotic (animal, fungal, or plant) origin. Alternatively, algorithms such as the one described in Smith and Smith (1992, Protein Engineering 5:35-51) could have been used to deal with primary sequence patterns and secondary structure gap penalties. All of the sequences disclosed in this application have lengths of at least 49 nucleotides, and no more than 12% uncalled bases (where N is recorded rather than A, C, G, or T).

As detailed in Karlin and Altschul (1993; Proc Natl Acad Sci 90:5873-5877), BLAST matches between a query sequence and a database sequence were evaluated statistically and only reported when they satisfied the threshold of  $10^{-25}$  for nucleotides and  $10^{-14}$  for peptides. Homology was also evaluated by product score calculated as follows: the % nucleotide or amino acid identity [between the query and reference sequences] in BLAST is multiplied by the % maximum possible BLAST score [based on the lengths of query and reference  
15 sequences] and then divided by 100. In comparison with hybridization procedures used in the laboratory, the electronic stringency for an exact match was set at 70, and the conservative lower limit for an exact match was set at approximately 40 (with 1-2% error due to uncalled bases).

The BLAST software suite, freely available sequence comparison algorithms (NCBI, Bethesda MD; <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>), includes various sequence analysis programs including "blastn" that is used to align nucleic acid molecules and BLAST 2 that is used for direct pairwise comparison of either  
25 nucleic or amino acid molecules. BLAST programs are commonly used with gap and other parameters set to default settings, e.g.: Matrix: BLOSUM62; Reward for match: 1; Penalty for mismatch: -2; Open Gap: 5 and Extension Gap: 2 penalties; Gap x drop-off: 50; Expect: 10; Word Size: 11; and Filter: on. Identity or similarity is measured over the entire length of a sequence or some smaller portion thereof. Brenner *et al.*  
30 (1998; Proc Natl Acad Sci 95:6073-6078, incorporated herein by reference) analyzed the BLAST for its ability to identify structural homologs by sequence identity and found 30% identity is a reliable threshold for sequence alignments of at least 150 residues and 40%, for alignments of at least 70 residues.

The polynucleotides of this application were compared with assembled consensus sequences or

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templates found in the LIFESEQ GOLD database. Component sequences from cDNA, extension, full length, and shotgun sequencing projects were subjected to PHRED analysis and assigned a quality score. All sequences with an acceptable quality score were subjected to various pre-processing and editing pathways to remove low quality 3' ends, vector and linker sequences, polyA tails, Alu repeats, mitochondrial and ribosomal sequences, and bacterial contamination sequences. Edited sequences had to be at least 50 bp in length, and low-information sequences and repetitive elements such as dinucleotide repeats, Alu repeats, and the like, were replaced by "Ns" or masked.

Edited sequences were subjected to assembly procedures in which the sequences were assigned to polynucleotide bins. Each sequence could only belong to one bin, and sequences in each bin were assembled to produce a template. Newly sequenced components were added to existing bins using BLAST and CROSSMATCH. To be added to a bin, the component sequences had to have a BLAST quality score greater than or equal to 150 and an alignment of at least 82% local identity. The sequences in each bin were assembled using PHRAP. Bins with several overlapping component sequences were assembled using DEEP PHRAP. The orientation of each template was determined based on the number and orientation of its component sequences.

Bins were compared to one another and those having local similarity of at least 82% were combined and reassembled. Bins having templates with less than 95% local identity were split. Templates were subjected to analysis by STITCHER/EXON MAPPER algorithms that analyze the probabilities of the presence of splice variants, alternatively spliced exons, splice junctions, differential expression of alternative spliced genes across tissue types or disease states, and the like. Assembly procedures were repeated periodically, and templates were annotated using BLAST against GenBank databases such as GBpri. An exact match was defined as having from 95% local identity over 200 base pairs through 100% local identity over 100 base pairs and a homolog match as having an E-value (or probability score) of  $\leq 1 \times 10^{-8}$ . The templates were also subjected to frameshift FASTx against GENPEPT, and homolog match was defined as having an E-value of  $\leq 1 \times 10^{-8}$ . Template analysis and assembly was described in USSN 09/276,534, filed March 25, 1999.

Following assembly, templates were subjected to BLAST, motif, and other functional analyses and categorized in protein hierarchies using methods described in USSN 08/812,290 and USSN 08/811,758, both filed March 6, 1997; in USSN 08/947,845, filed October 9, 1997; and in USSN 09/034,807, filed March 4, 1998. Then templates were analyzed by translating each template in all three forward reading frames and searching each translation against the PFAM database of hidden Markov model-based protein families and domains using the HMMER software package (Washington University School of Medicine, St. Louis MO; <http://pfam.wustl.edu/>).

The polynucleotide was further analyzed using MACDNASIS PRO software (Hitachi Software

Engineering), and LASERGENE software (DNASTAR) and queried against public databases such as the GenBank rodent, mammalian, vertebrate, prokaryote, and eukaryote databases, SwissProt, BLOCKS, PRINTS, PFAM, and Prosite.

## V DESCRIPTION OF KNOWN CARDIAC MUSCLE-ASSOCIATED GENES

Twelve known cardiac muscle-associated genes were selected to identify novel polynucleotides that are closely associated with cardiac muscle function. These known genes were atrial regulatory myosin, ventricular myosin alkali light chain, cardiac troponin, cardiac ventricular myosin, cardiodilatin, creatine kinase M, myoglobin, natriuretic peptide precursor, sarcomeric mitochondrial creatine kinase, telethonin, titin, and urocortin.

Brief descriptions of the known cardiac muscle-associated genes and their expression in cardiac disorders are presented below.

GENE	DESCRIPTION AND REFERENCES
atrial regulatory myosin	Predominant regulatory myosin light chain isoform in adult atrial muscle. Differentially expressed in cardiovascular development and disease. Fewell et al. (1998) J Clin Invest 101:2630-2639; Hailstones et al. (1992) J. Biol. Chem. 267:23295-23300.
ventricular myosin alkali light chain	Muscle fiber protein. Differentially expressed in altered cardiovascular function and in myocardial hypertrophy. Morano et al. (1997) J Mol Cell Cardiol 29:1177-1187.
troponin	Marker of cardiac injury. Feng et al. (1998) Am J Clin Pathol 110:70-77; Luscher et al. (1998) Cardiology 89:222-228; and Kost et al. (1998) Arch Pathol Lab Med 122:245-251.
cardiac ventricular myosin	Muscle fiber protein. Expressed in cardiac remodeling after myocardial infarction. Differentially expressed in altered cardiovascular function. Trahair et al. (1993) J Mol Cell Cardiol 25:577-585.
cardiodilatin	Differentially expressed following myocardial infarction. Induces vasorelaxation. Gidh-Jain et al. (1998) J Mol Cell Cardiol 30:627-637; Magga et al. (1998) Ann Med 30(S1):39-45.
creatine kinase M	Marker of cardiac injury. Feng, <u>supra</u> ; Luscher, <u>supra</u> ; and Kost, <u>supra</u> .
myoglobin	Marker of cardiac injury. Feng, <u>supra</u> ; Luscher, <u>supra</u> ; and Kost, <u>supra</u> .
natriuretic peptide precursor	See cardiodilatin.
sarcomeric mitochondrial creatine kinase	Essential enzyme in energy metabolism, particularly in tissue with high energy requirements. Klein et al. (1991) J Biol Chem 266:18058-18065; Qin et al. (1997) J Biol Chem 272:25210-25216.
telethonin	Sarcomeric protein of heart and skeletal muscle. Valle et al. (1997) FEBS Lett. 415:163-168; Mayans et al. (1998) Nature 395:863-869.

<b>titin</b>	<b>Muscle fiber protein. Temporal and spatial control of sarcomere assembly. Differentially expressed after atrial fibrillation. Ausma et al. (1997) Am J Pathol 151:985-997; Mayans, <u>supra</u>.</b>
<b>urocortin</b>	<b>Stimulates atrial natriuretic peptide secretion. Expression increased following cardiac injury. Protects cardiac myocytes from hypoxic death. Ikeda et al. (1998) Biochem. Biophys Res Commun 250:298-304; Asaba et al. (1998) Brain Res 806:95-103; and Okosi et al. (1998) Neuropeptides 32:167-171.</b>

## VI CO-EXPRESSION AMONG KNOWN MARKER GENES AND NOVEL POLYNUCLEOTIDES

GBA identified 48 novel polynucleotides from a total of 45,233 assembled sequences that showed strong expression and association with the known cardiac muscle-associated genes. The process was reiterated until the number of polynucleotides was reduced to the final 48 polynucleotides shown below. Each of the 48 polynucleotides is co-expressed with at least one of the twelve known genes with a  $p$ -value of less than  $10^{-05}$ .

The co-expression of the novel polynucleotides and the known genes are shown in Table 1-1, 1-2, and 1-3. The novel polynucleotides are listed along the top of the table by their SEQ ID NO, and the known genes, by their names in the rows down the side of the table. The entries in the table are the negative log of the  $p$ -value ( $-\log p$ ) for the co-expression of two sequences. For each polynucleotide, the  $p$ -value is the probability that the observed co-expression is due to chance, using the Fisher Exact Test.

The highest co-expression value is obtained when the highest  $p$ -value found in a vertical column below the SEQ ID NO (clone number) is correlated with the name of a known marker gene listed for that row. For example, SEQ ID NO:4, has a  $p$ -value of 19 as it co-expresses with cardiac ventricular myosin. This highly significant  $p$ -value substantiates that SEQ ID NO:4, SEQ ID NO:49, and an antibody which specifically binds SEQ ID NO:49 can be used as surrogate markers for cardiac ventricular myosin in a diagnostic assay for myocardial infarction.

The data above can be summarized by reducing it to a single highest co-expression ( $-\log p$ ) value for each intersecting known gene and unknown polynucleotide and naming at least one disorder associated with expression of the known gene. A summary table is shown below:

SEQ ID NO	p-value	Gene	Disorder
1	7	atrial regulatory myosin	cardiac injury
2	6	natriuretic peptide precursor	myocardial infarction
3	7	telethonin	atrial fibrillation
4	19	cardiac ventricular myosin	myocardial infarction
5	9	creatine kinase M	cardiac injury
6	11	titin	atrial fibrillation
7	10	troponin	cardiac injury
8	6	natriuretic peptide precursor	myocardial infarction
9	6	urocortin	myocardial infarction
10	12	telethonin	atrial fibrillation

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	11	8	creatine kinase M	cardiac injury
	12	9	atrial regulatory myosin	cardiac injury
	13	22	titin	atrial fibrillation
	14	8	ventricular myosin alkali light chain	myocardial hypertrophy
5	15	10	titin	atrial fibrillation
	16	7	titin	atrial fibrillation
	17	8	telethonin	atrial fibrillation
	18	6	urocortin	myocardial infarction
	19	11	creatine kinase M	cardiac injury
10	20	13	myoglobin	cardiac injury
	21	10	ventricular myosin alkali light chain	myocardial hypertrophy
	22	10	troponin	cardiac injury
	23	11	titin	atrial fibrillation
	24	7	ventricular myosin alkali light chain	myocardial hypertrophy
15	25	9	ventricular myosin alkali light chain	myocardial hypertrophy
	26	18	creatine kinase M	cardiac injury
	27	19	ventricular myosin alkali light chain	myocardial hypertrophy
	28	21	creatine kinase M	cardiac injury
	29	5	sarcomeric mitoch. creatine kinase	<del>hypertension</del>
20	30	15	myoglobin	cardiac injury
	31	7	telethonin	atrial fibrillation
	32	8	creatine kinase M	cardiac injury
	33	11	titin	atrial fibrillation
	34	9	atrial regulatory myosin	cardiac injury
25	35	8	creatine kinase M	cardiac injury
	36	7	cardiac ventricular myosin	myocardial infarction
	37	16	myoglobin	cardiac injury
	38	11	myoglobin	cardiac injury
	39	21	creatine kinase M	cardiac injury
30	40	11	creatine kinase M	cardiac injury
	41	20	creatine kinase M	cardiac injury
	42	8	titin	atrial fibrillation
	43	6	cardiac ventricular myosin	myocardial infarction
	44	7	cardiodilantin	myocardial infarction
35	45	10	telethonin	atrial fibrillation
	46	11	creatine kinase M	cardiac injury
	47	9	atrial regulatory myosin	cardiac injury
	48	9	telethonin	atrial fibrillation

\* p-value (- log p) = 5 is highly significant

**40 VII DESCRIPTION OF THE POLYNUCLEOTIDES IDENTIFIED USING GBA**

Using the method of Walker (supra), 48 polynucleotides that exhibit strong association, or co-expression, with cardiac muscle-associated genes have been identified.

Polynucleotides comprising the nucleic acid sequences of SEQ ID NOs:1-48 of the present invention were first identified as Incyte Clones 2045674, 188552, 465676, 3601719, 305781, 971441, 3445829, 189299, 45 2396760, 919893, 2837330, 1737459, 058201, 767447, 5449893, 2951269, 282977, 3178454, 3563859,



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985730, 3684987, 986166, 1887508, 1006416, 975169, 4152861, 986464, 118472, 1314633, 1997439, 2638878, 3795510, 1413537, 1623157, 3009303, 3434460, 5022769, 944140, 3445829, 3016490, 4151935, 3719652, 3046106, 3012947, 466761, 1644171, 3009806, and 5578191, respectively; and assembled according to Example III. As described in Example IV, BLAST and other motif searches were performed for each sequence. SEQ ID NOs:1-48 were translated, and identity with known sequences was sought. Proteins comprising SEQ ID NOs:49-62 were also analyzed using BLAST and other motif search tools as disclosed in Example VI. The details of the various analyses are described in Table 2.

### **VIII HYBRIDIZATION TECHNOLOGIES AND ANALYSES**

#### **Immobilization of Polynucleotides on a Substrate**

The polynucleotides are applied to a substrate by one of the following methods. A mixture of polynucleotides is fractionated by gel electrophoresis and transferred to a nylon membrane by capillary transfer. Alternatively, the polynucleotides are individually ligated to a vector and inserted into bacterial host cells to form a library. The polynucleotides are then arranged on a substrate by one of the following methods. In the first method, bacterial cells containing individual clones are robotically picked and arranged on a nylon membrane. The membrane is placed on LB agar containing selective agent (carbenicillin, kanamycin, ampicillin, or chloramphenicol depending on the vector used) and incubated at 37C for 16 hr. The membrane is removed from the agar and consecutively placed colony side up in 10% SDS, denaturing solution (1.5 M NaCl, 0.5 M NaOH), neutralizing solution (1.5 M NaCl, 1 M Tris-HCl, pH 8.0), and twice in 2xSSC for 10 min each. The membrane is then UV irradiated in a STRATALINKER UV-crosslinker (Stratagene).

In the second method, polynucleotides are amplified from bacterial vectors by thirty cycles of PCR using primers complementary to vector sequences flanking the insert. PCR amplification increases a starting concentration of 1-2 ng nucleic acid to a final quantity greater than 5  $\mu$ g. Amplified nucleic acids from about 400 bp to about 5000 bp in length are purified using SEPHACRYL-400 beads (APB). Purified nucleic acids are arranged on a nylon membrane manually or using a dot/slot blotting manifold and suction device and are immobilized by denaturation, neutralization, and UV irradiation as described above. Purified nucleic acids are robotically arranged and immobilized on polymer-coated glass slides using the procedure described in USPN 5,807,522. Polymer-coated slides are prepared by cleaning glass microscope slides (Corning, Acton MA) by ultrasound in 0.1% SDS and acetone, etching in 4% hydrofluoric acid (VWR Scientific Products, West Chester PA), coating with 0.05% aminopropyl silane (Sigma-Aldrich) in 95% ethanol, and curing in a 110C oven. The slides are washed extensively with distilled water between and after treatments. The nucleic acids are arranged on the slide and then immobilized by exposing the array to UV irradiation using a STRATALINKER UV-crosslinker (Stratagene). Arrays are then washed at room temperature in 0.2% SDS and rinsed three times in distilled water. Non-specific binding sites are blocked by incubation of arrays in 0.2% casein in phosphate

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buffered saline (PBS; Tropix, Bedford MA) for 30 min at 60C; then the arrays are washed in 0.2% SDS and rinsed in distilled water as before.

#### Probe Preparation for Membrane Hybridization

Hybridization probes derived from the polynucleotides of the Sequence Listing are employed for screening cDNAs, mRNAs, or genomic DNA in membrane-based hybridizations. Probes are prepared by diluting the polynucleotides to a concentration of 40-50 ng in 45  $\mu$ l TE buffer, denaturing by heating to 100C for five min, and briefly centrifuging. The denatured polynucleotide is then added to a REDIPRIME tube (APB), gently mixed until blue color is evenly distributed, and briefly centrifuged. Five  $\mu$ l of [<sup>32</sup>P]dCTP is added to the tube, and the contents are incubated at 37C for 10 min. The labeling reaction is stopped by adding 5  $\mu$ l of 0.2M EDTA, and probe is purified from unincorporated nucleotides using a PROBEQUANT G-50 microcolumn (APB). The purified probe is heated to 100C for five min, snap cooled for two min on ice, and used in membrane-based hybridizations as described below.

#### Probe Preparation for Polymer Coated Slide Hybridization

Hybridization probes derived from mRNA isolated from samples are employed for screening polynucleotides of the Sequence Listing in array-based hybridizations. Probe is prepared using the GEMbright kit (Incyte Genomics) by diluting mRNA to a concentration of 200 ng in 9  $\mu$ l TE buffer and adding 5  $\mu$ l 5x buffer, 1  $\mu$ l 0.1 M DTT, 3  $\mu$ l Cy3 or Cy5 labeling mix, 1  $\mu$ l RNase inhibitor, 1  $\mu$ l reverse transcriptase, and 5  $\mu$ l 1x yeast control mRNAs. Yeast control mRNAs are synthesized by in vitro transcription from noncoding yeast genomic DNA (W. Lei, unpublished). As quantitative controls, one set of control mRNAs at 0.002 ng, 0.02 ng, 0.2 ng, and 2 ng are diluted into reverse transcription reaction mixture at ratios of 1:100,000, 1:10,000, 1:1000, and 1:100 (w/w) to sample mRNA respectively. To examine mRNA differential expression patterns, a second set of control mRNAs are diluted into reverse transcription reaction mixture at ratios of 1:3, 3:1, 1:10, 10:1, 1:25, and 25:1 (w/w). The reaction mixture is mixed and incubated at 37C for two hr. The reaction mixture is then incubated for 20 min at 85C, and probes are purified using two successive CHROMA SPIN+TE 30 columns (Clontech, Palo Alto CA). Purified probe is ethanol precipitated by diluting probe to 90  $\mu$ l in DEPC-treated water, adding 2  $\mu$ l 1mg/ml glycogen, 60  $\mu$ l 5 M sodium acetate, and 300  $\mu$ l 100% ethanol. The probe is centrifuged for 20 min at 20,800xg, and the pellet is resuspended in 12  $\mu$ l resuspension buffer, heated to 65C for five min, and mixed thoroughly. The probe is heated and mixed as before and then stored on ice. Probe is used in high density array-based hybridizations as described below.

#### Membrane-based Hybridization

Membranes are pre-hybridized in hybridization solution containing 1% Sarkosyl and 1x high phosphate buffer (0.5 M NaCl, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 5 mM EDTA, pH 7) at 55C for two hr. The probe, diluted in 15 ml fresh hybridization solution, is then added to the membrane. The membrane is hybridized with the probe at 55C for

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16 hr. Following hybridization, the membrane is washed for 15 min at 25C in 1mM Tris (pH 8.0), 1% Sarkosyl, and four times for 15 min each at 25C in 1mM Tris (pH 8.0). To detect hybridization complexes, XOMAT-AR film (Eastman Kodak, Rochester NY) is exposed to the membrane overnight at -70C, developed, and examined visually.

5 Polymer Coated Slide-based Hybridization

Probe is heated to 65C for five min, centrifuged five min at 9400 rpm in a 5415C microcentrifuge (Eppendorf Scientific, Westbury NY), and then 18  $\mu$ l are aliquoted onto the array surface and covered with a coverslip. The arrays are transferred to a waterproof chamber having a cavity just slightly larger than a microscope slide. The chamber is kept at 100% humidity internally by the addition of 140  $\mu$ l of 5xSSC in a corner of the chamber. The chamber containing the arrays is incubated for about 6.5 hr at 60C. The arrays are washed for 10 min at 45C in 1xSSC, 0.1% SDS, and three times for 10 min each at 45C in 0.1xSSC, and dried.

Hybridization reactions are performed in absolute or differential hybridization formats. In the absolute hybridization format, probe from one sample is hybridized to array elements, and signals are detected after hybridization complexes form. Signal strength correlates with probe mRNA levels in the sample. In the differential hybridization format, differential expression of a set of genes in two biological samples is analyzed. Probes from the two samples are prepared and labeled with different labeling moieties. A mixture of the two labeled probes is hybridized to the array elements, and signals are examined under conditions in which the emissions from the two different labels are individually detectable. Elements on the array that are hybridized to equal numbers of probes derived from both biological samples give a distinct combined fluorescence (Shalon WO95/35505).

Hybridization complexes are detected with a microscope equipped with an INNOVA 70 mixed gas 10 W laser (Coherent, Santa Clara CA) capable of generating spectral lines at 488 nm for excitation of Cy3 and at 632 nm for excitation of Cy5. The excitation laser light is focused on the array using a 20X microscope objective (Nikon, Melville NY). The slide containing the array is placed on a computer-controlled X-Y stage on the microscope and raster-scanned past the objective with a resolution of 20 micrometers. In the differential hybridization format, the two fluorophores are sequentially excited by the laser. Emitted light is split, based on wavelength, into two photomultiplier tube detectors (PMT R1477, Hamamatsu Photonics Systems, Bridgewater NJ) corresponding to the two fluorophores. Appropriate filters positioned between the array and the photomultiplier tubes are used to filter the signals. The emission maxima of the fluorophores used are 565 nm for Cy3 and 650 nm for Cy5. The sensitivity of the scans is calibrated using the signal intensity generated by the yeast control mRNAs added to the probe mix. A specific location on the array contains a complementary DNA sequence, allowing the intensity of the signal at that location to be correlated with a weight ratio of hybridizing species of 1:100,000.

The output of the photomultiplier tube is digitized using a 12-bit RTI-835H analog-to-digital (A/D) conversion board (Analog Devices, Norwood MA) installed in an IBM-compatible PC computer. The digitized data are displayed as an image where the signal intensity is mapped using a linear 20-color transformation to a pseudocolor scale ranging from blue (low signal) to red (high signal). The data is also analyzed quantitatively.

- 5 Where two different fluorophores are excited and measured simultaneously, the data are first corrected for optical crosstalk (due to overlapping emission spectra) between the fluorophores using the emission spectrum for each fluorophore. A grid is superimposed over the fluorescence signal image such that the signal from each spot is centered in each element of the grid. The fluorescence signal within each element is then integrated to obtain a numerical value corresponding to the average intensity of the signal. The software used for signal  
10 analysis is the GEMTOOLS program (Incyte Genomics).

## IX TRANSCRIPT IMAGING

The transcript image performed using the LIFESEQ GOLD database (Aug00rel, Incyte Genomics) allowed assessment of the relative abundance of expressed polynucleotides in one or more cDNA libraries. Criteria for transcript imaging include category, number of cDNAs per library, description of the library, and the like

- 15 All sequences and cDNA libraries in the LIFESEQ database were categorized by system, organ/tissue and cell type. The categories included cardiovascular system, connective tissue, digestive system, embryonic structures, endocrine system, exocrine glands, female and male reproductive, germ cells, hemic/immune system, liver, musculoskeletal system, nervous system, pancreas, respiratory system, sense organs, skin, stomatognathic system, unclassified/mixed, and the urinary tract. For each category, the number of libraries in which the sequence was expressed were counted and shown over the total number of libraries in that category. In some transcript images, all normalized or pooled libraries, which have high copy number sequences removed prior to processing, and all mixed or pooled tissues, which are considered non-specific in that they contain  
20 more than one tissue type or more than one subject's tissue, can be excluded from the analysis. Cell lines and/or fetal tissue data can also be disregarded unless the elucidation of inherited disorders would be furthered by their inclusion in the analysis.

- 25 For diagnostic purposes, the standards to which biopsied samples would be compared are: cytologically normal, non-diseased samples versus samples which had been diagnosed with specific cardiac disorders including, but not limited to, atherosclerosis, arteriosclerosis, atrial fibrillation, cancer (myxoma) and complications of cancer, cardiac injury, congestive heart failure, coronary artery disease, hypertension, hypertrophic cardiomyopathy, myocardial hypertrophy, myocardial infarction, and plaque.

30 For purposes of example, the transcript images for SEQ ID NOs:29 and 44 are shown below. The first column shows library name; the second column, the number of cDNAs sequenced in that library; the third

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column, the description of the library; and the fourth column, absolute abundance of the transcript in the library.

**SEQ ID NO:29** (Category: Cardiovascular\*)

<u>Library</u>	<u>cDNA</u>	<u>Description</u>	<u>Abundance</u>	<u>%Abundance</u>
HEARNOT06	3685	heart, hypertension, 44M	2	0.0543
HEARFET05	2524	heart, fetal, M	1	0.0396
<u>HEARFET02</u>	6919	heart, hypoplastic left, fetal, 23wM	1	0.0145

\*No libraries were removed from the analysis.

**SEQ ID NO:44** (Category: Cardiovascular\*)

<u>Library</u>	<u>cDNA</u>	<u>Description</u>	<u>Abundance</u>	<u>%Abundance</u>
HEALDIT02	4171	left ventricle, mw/myocardial infarction, 56M	1	0.0240
<u>HEARFET02</u>	6919	heart, hypoplastic left, fetal, 23wM	1	0.0145

\*Normalized and pooled libraries were removed from the analysis.

SEQ ID NOs:29 and 44 were differentially expressed when compared by percent abundance to useful standards (i.e., the up-regulation of SEQ ID NOs:29 in heart tissue of a deceased victim who was shot to death is not a comparison that would be made in a diagnostic setting). More importantly, these sequences are not differentially expressed in any normal tissue or diagnostic of any other cardiac disorder.

The differential expression of SEQ ID NOs:29, and 44, respectively, in tissue associated with hypertension and myocardial infarction, respectively, supports the use of the sequences as a surrogate markers for sarcomeric mitochondrial creatine kinase and cardiodilantin, respectively. These transcript images verify GBA analysis (see Example VI above).

**X COMPLEMENTARY MOLECULES**

The complement of the novel polynucleotide, from about 5 bp (e.g., a PNA) to about 5000 bp (e.g., the complement of a cDNA insert), are used to detect or inhibit gene expression. These molecules are selected using LASERGENE software (DNASTAR). Detection is described in Example VIII. To inhibit transcription by preventing promoter binding, the complementary molecule is designed to bind to the most unique 5' sequence and includes nucleotides of the 5' UTR upstream of the initiation codon of the open reading frame. Complementary molecules include genomic sequences (such as enhancers or introns) and are used in "triple helix" base pairing to compromise the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. To inhibit translation, a complementary molecule is designed to prevent ribosomal binding to the mRNA encoding the protein.

Complementary molecules are placed in expression vectors and used to transform a cell line to test efficacy; into an organ, tumor, synovial cavity, or the vascular system for transient or short term therapy; or into a stem cell, zygote, or other reproducing lineage for long term or stable gene therapy. Transient expression

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lasts for a month or more with a non-replicating vector and for three months or more if appropriate elements for inducing vector replication are used in the transformation/expression system.

Stable transformation of appropriate dividing cells with a vector encoding the complementary molecule produces a transgenic cell line, tissue, or organism (USPN 4,736,866). Those cells that assimilate and replicate sufficient quantities of the vector to allow stable integration also produce enough complementary molecules to compromise or entirely eliminate activity of the polynucleotide encoding the protein.

#### **XI PROTEIN EXPRESSION**

Expression and purification of the protein are achieved using either a cell expression system or an insect cell expression system. The pUB6/V5-His vector system (Invitrogen, Carlsbad CA) is used to express protein in CHO cells. The vector contains the selectable *bsd* gene, multiple cloning sites, the promoter/enhancer sequence from the human ubiquitin C gene, a C-terminal V5 epitope for antibody detection with anti-V5 antibodies, and a C-terminal polyhistidine (6xHis) sequence for rapid purification on PROBOND resin (Invitrogen). Transformed cells are selected on media containing blasticidin.

Spodoptera frugiperda (Sf9) insect cells are infected with recombinant Autographica californica nuclear polyhedrosis virus (baculovirus). The polyhedrin gene is replaced with the polynucleotide by homologous recombination and the polyhedrin promoter drives transcription. The protein is synthesized as a fusion protein with 6xhis which enables purification as described above. Purified protein is used in the following activity and to make antibodies.

#### **XII PRODUCTION OF ANTIBODIES**

The protein is purified using polyacrylamide gel electrophoresis and used to immunize mice or rabbits. Antibodies are produced using the protocols below. Alternatively, the amino acid sequence of the expressed protein is analyzed using LASERGENE software (DNASTAR) to determine regions of high antigenicity. An antigenic epitope, usually found near the C-terminus or in a hydrophilic region is selected, synthesized, and used to raise antibodies. Typically, epitopes of about 15 residues in length are produced using an ABI 431A peptide synthesizer (ABI) using FMOC-chemistry and coupled to KLH (Sigma-Aldrich) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester to increase antigenicity.

Rabbits are immunized with the epitope-KLH complex in complete Freund's adjuvant. Immunizations are repeated at intervals thereafter in incomplete Freund's adjuvant. After a minimum of seven weeks for mouse or twelve weeks for rabbit, antisera are drawn and tested for antipeptide activity. Testing involves binding the peptide to plastic, blocking with 1% bovine serum albumin, reacting with rabbit antisera, washing, and reacting with radio-iodinated goat anti-rabbit IgG. Methods well known in the art are used to determine antibody titer and the amount of complex formation.

#### **XIII PURIFICATION OF NATURALLY OCCURRING PROTEIN USING SPECIFIC ANTIBODIES**

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Naturally occurring or recombinant protein is purified by immunoaffinity chromatography using antibodies which specifically bind the protein. An immunoaffinity column is constructed by covalently coupling the antibody to CNBr-activated SEPHAROSE resin (APB). Media containing the protein is passed over the immunoaffinity column, and the column is washed using high ionic strength buffers in the presence of detergent to allow preferential absorbance of the protein. After coupling, the protein is eluted from the column using a buffer of pH 2-3 or a high concentration of urea or thiocyanate ion to disrupt antibody/protein binding, and the protein is collected.

#### XIV SCREENING MOLECULES FOR SPECIFIC BINDING USING POLYNUCLEOTIDE OR PROTEIN

The polynucleotide, or fragments thereof, or the protein, or portions thereof, are labeled with  $^{32}\text{P}$ -dCTP, Cy3-dCTP, or Cy5-dCTP (APB), or with BIODIPY or FITC (Molecular Probes, Eugene OR), respectively. Libraries of candidate molecules or compounds previously arranged on a substrate are incubated in the presence of composition, a labeled polynucleotide or protein. After incubation under conditions for either a nucleic acid or amino acid sequence, the substrate is washed, and any position on the substrate retaining label, which indicates specific binding or complex formation, is assayed, and the ligand is identified. Data obtained using different concentrations of the nucleic acid or protein are used to calculate affinity between the labeled nucleic acid or protein and the bound molecule.

#### XV TWO-HYBRID SCREEN

A yeast two-hybrid system, MATCHMAKER LexA Two-Hybrid system (Clontech Laboratories, Palo Alto CA), is used to screen for peptides that bind the protein of the invention. A polynucleotide encoding the protein is inserted into the multiple cloning site of a pLexA vector, ligated, and transformed into *E. coli*. cDNA, prepared from mRNA, is inserted into the multiple cloning site of a pB42AD vector, ligated, and transformed into *E. coli* to construct a cDNA library. The pLexA plasmid and pB42AD-cDNA library constructs are isolated from *E. coli* and used in a 2:1 ratio to co-transform competent yeast EGY48[p8op-lacZ] cells using a polyethylene glycol/lithium acetate protocol. Transformed yeast cells are plated on synthetic dropout (SD) media lacking histidine (-His), tryptophan (-Trp), and uracil (-Ura), and incubated at 30C until the colonies have grown up and are counted. The colonies are pooled in a minimal volume of 1x TE (pH 7.5), replated on SD/-His/-Leu/-Trp/-Ura media supplemented with 2% galactose (Gal), 1% raffinose (Raf), and 80 mg/ml 5-bromo-4-chloro-3-indolyl  $\beta$ -d-galactopyranoside (X-Gal), and subsequently examined for growth of blue colonies. Interaction between expressed protein and cDNA fusion proteins activates expression of a LEU2 reporter gene in EGY48 and produces colony growth on media lacking leucine (-Leu). Interaction also activates expression of  $\beta$ -galactosidase from the p8op-lacZ reporter construct that produces blue color in colonies grown on X-Gal.

Positive interactions between expressed protein and cDNA fusion proteins are verified by isolating

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individual positive colonies and growing them in SD/-Trp/-Ura liquid medium for 1 to 2 days at 30C. A sample of the culture is plated on SD/-Trp/-Ura media and incubated at 30C until colonies appear. The sample is replica-plated on SD/-Trp/-Ura and SD/-His/-Trp/-Ura plates. Colonies that grow on SD containing histidine but not on media lacking histidine have lost the pLexA plasmid. Histidine-requiring colonies are grown on  
5 SD/Gal/Raf/X-Gal/-Trp/-Ura, and white colonies are isolated and propagated. The pB42AD-cDNA plasmid, which contains a polynucleotide encoding a protein that physically interacts with the protein, is isolated from the yeast cells and characterized.

All patents and publications mentioned in the specification are incorporated by reference herein.

Various modifications and variations of the described method and system of the invention will be apparent to  
10 those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.